

**REMARKS**

The Office Action dated December 18, 2003 presents the examination of claims 1 and 3-6. Claim 4 is canceled. Claims 1, 3, 5, and 6 are amended. Support for the recitation of "inhibiting the activation of a substance" is found in the specification, such as on page 3, lines 2-6. Support for the recitation of "brain natriuretic peptides" is found on page 3, line 23 of the specification. Claims 12 and 13 are added. Support for the recitation of "whole blood or blood plasma" is found on page 4, lines 16-17 of the specification. No new matter is inserted into the application.

***Rejection under 35 U.S.C. § 102***

The Examiner rejects claims 1 and 3-6 under 35 U.S.C. § 102(b) for allegedly being anticipated by Lindberg et al. (*Pharmacology & Toxicology*) and Clerico et al. (*Clinical Chemistry*). Claim 4 is canceled, thus rendering rejection thereof moot. Applicants respectfully traverse the rejection applied to the pending claims. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

The Present Invention

Exhibit 1 attached hereto is a schematic diagram depicting the present invention. The present invention relates to a method of inhibiting the activation of a substance degrading BNP in a specimen by using a container made of or coated with a material selected from the group consisting of silicone and plastics.

The specimen (such as whole blood or blood plasma) includes not only BNP, but also substances (such as proteases) which degrade BNP. It is known in the art that proteases have an "active" form and an "inactive" form. Proteases only have the ability to degrade BNP when they exist in "active" form.

When blood is collected from a donor, the proteases which have the ability to degrade BNP exist in the "inactive" form. See, box labeled "Protease (INACTIVE)" in Exhibit 1. The present inventors surprisingly found that when blood is placed into a glass container, the glass container converts the protease into active form. See, box labeled "Protease (ACTIVE)" in Exhibit 1. In fact, at the filing date of the present application, those skilled in the art deemed it necessary to add protease inhibitors (such as aprotinin) to blood in order to inhibit the degradation of ANP by the proteases. See, step 2 of Exhibit 1.

The present inventors found that BNP in specimens are degraded

when placed into a glass tube, and that the degradation of BNP could be suppressed by placing the specimen into a container made of or coated with silicone or plastics, without the addition of protease inhibitors such as aprotinin. See, Examples 1-8 in the specification.

Based upon the above, the present inventors found that the activation of a substance degrading BNP in a specimen can be suppressed by using a container made of or coated with a material selected from the group consisting of silicone or plastics. See, step 1 of Exhibit 1. Unlike in the prior art, the addition of a protease inhibitor is not necessary.

For example, in Shimizu et al., *Clinica Chemica Acta*, 305:181-186 (2001) (attached hereto as Exhibit 2), the present inventors show that the activity of a protease, kallikrein, in blood plasma stored in a glass tube was increased, whereas the activity of kallikrein was negligible in samples stored in PET tubes in the absence of protease inhibitors. See, Figure 4, page 184. Exhibit 2 shows that glass containers can convert an "inactive" form of a substance degrading mammalian natriuretic peptide into an "active" form. This finding explains why BNP activity in the absence of aprotinin decreases in a glass tube but not in a silicon-coated glass tube or a plastic tube. See, Examples 1-3 of the

specification.

In Shimizu et al., *Clinica Chemica Acta*, 285:169-172 (1999), attached hereto as Exhibit 3), the present inventors show that BNP in PET tubes with aprotinin is less stable than BNP in PET tubes without aprotinin. See, Figure 1, page 171. This finding shows that the selection of the appropriate container (i.e., containers made of or coated with a material selected from the group consisting of silicone and plastics) is more important than the addition of a protease inhibitor to the stability of BNP.

#### The prior art

The cited references disclose that inhibiting agents must be added to the specimen for suppressing the degradation of ANP. Both references fail to disclose or teach that the activation of a substance degrading BNP in specimens can be suppressed by using a container made of or coated with a material selected from the group consisting of silicone and plastics. Therefore, the present invention is different from the references cited by the Examiner.

Specifically, Lindberg et al. describes the addition of **purified** atrial natriuretic peptide (ANP) to siliconized glass tubes and plastic tubes. Since the samples studied by Lindberg et al. are not, for example, bodily samples, there are **no** proteases

normally present in specimens which would degrade the mammalian natriuretic peptides in their samples. Thus, Lindberg et al. did not need to care about or investigate the degradation of ANP by proteases. In fact, Lindberg et al. merely discloses that the adsorption of ANP to containers can be easily avoided by the addition of HSA (human serum albumin). Nowhere does Lindberg et al. disclose or teach that the activation of a substance degrading BNP (i.e., a protease) in a specimen can be suppressed by placing the specimen into a container made of or coated with a material selected from the group consisting of silicone and plastics.

The Examiner asserts that Lindberg et al. teaches loss of recovery of ANP at different concentrations in different containers including silicon glass and coated polymers, as well as ANP in solution with and without degradation inhibiting compounds. Such examples are irrelevant to the present invention. As noted above, the ANP used by Lindberg et al. is in **purified** form. Thus, there are no substances in Lindberg's samples which would degrade ANP normally found in specimens such as whole blood. The skilled artisan could not extrapolate from Lindberg et al. that the activation of a substance degrading BNP in specimens can be suppressed by using a container made of or coated with a material selected from the group consisting of silicone and plastics, simply

because there are no substances present which would degrade BNP or ANP in Lindberg's samples. For this reason, Lindberg et al. fails to anticipate the present invention.

Clerico et al. discloses that the degradation of ANP can be avoided by (1) separating plasma from a blood sample, (2) adding a protease inhibitor, aprotinin, and (3) storing the plasma at low temperatures. As noted by the Examiner, Clerico discloses a sample without aprotinin (page 1629, first column). However, unlike the present invention, Clerico et al. concludes that aprotinin is **needed** for inhibiting the degradation of ANP.

On the other hand, the inventors of the present application show that the degradation of BNP can be avoided by using containers made of or coated with a material selected from the group consisting of silicone and plastics, even in the absence of protease inhibitors. See, Examples 1-3 of the specification. As mentioned above, aprotinin can inhibit the degradation of ANP by the "active" form of proteases. The present inventors discovered that containers such as a silicon-coated glass tube or a plastic tube can inhibit the conversion of the "inactive" form of proteases into the "active" form. For these reasons, the method of Clerico et al. and that of the present application are fundamentally different.

In summary, Lindberg et al. and Clerico et al. fail to anticipate the present invention under 35 U.S.C. § 102. Withdrawal of the instant rejection is therefore respectfully requested.

***Rejection under 35 U.S.C. § 112, second paragraph***

The Examiner rejects claims 3 and 4 under 35 U.S.C. § 112, second paragraph for allegedly being indefinite. Claim 4 is canceled, thus rendering rejection thereof moot. Applicants respectfully traverse the rejection applied to pending claim 3. Reconsideration of the claim and withdrawal of the instant rejection are respectfully requested.

The Examiner notes that claim 3 depends from claim 2, which was formally canceled. Claim 3 is amended to depend from claim 1. Thus, the instant rejection is overcome.

***Conclusion***

Applicants respectfully submit that the pending claims define patentable subject matter such that this application should be placed into condition for allowance. Early and favorable action on the merits of the present application is thereby requested.

If there are any minor matters precluding allowance of the present application which may be resolved by a telephone

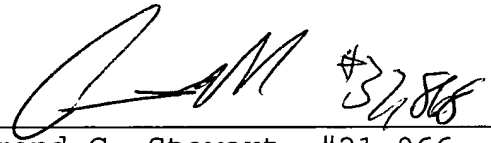
Appl. No. 09/530,013

discussion, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at (703) 205-8000.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

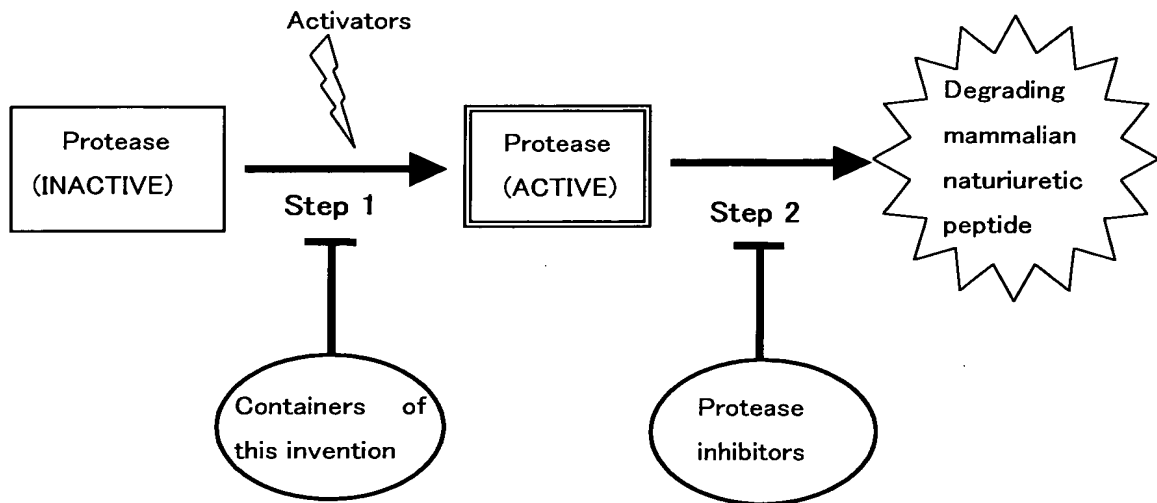
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**Figure**



## Degradation of human brain natriuretic peptide (BNP) by contact activation of blood coagulation system

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### Abstract

Brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) were added to venous blood samples from healthy volunteers, and incubated in tubes made of various materials. The residual immunoreactivity was measured with radioimmunoassay for BNP and ANP. In blood samples stored in glass tubes, immunoreactivity of ANP was more stable than that of BNP. In siliconized glass or PET tubes, however, BNP immunoreactivity was more stable than ANP. The activation of blood coagulation factors was evaluated from the kallikrein activity in plasma. Kallikrein activity was increased in plasma stored in glass tube while it was negligible in plasma stored in siliconized glass or PET tubes. In kaolin-activated plasma, more rapid BNP degradation and higher kallikrein activity were observed. Our results indicated that the blood coagulation factors, especially kallikrein, played an important role in digestion of BNP. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Atrial natriuretic peptide (ANP); Brain natriuretic peptide (BNP); Kallikrein; Blood coagulation factor; Contact activation system

### 1. Introduction

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are cardiac hormones, which are synthesized and secreted into the bloodstream mainly from the atria and ventricles of the heart, respectively [1–3]. Since the plasma concentrations of both hormones are increased with the decline of heart function, measurements of their concentrations in blood, especially BNP, are useful for the diagnosis and prognosis of acute myocardial infarction or heart

failure [3–7]. Highly sensitive assay systems have allowed the measurement of both peptides for clinical applications [8].

Since ANP and BNP are small peptides consisting of 28 and 32 amino acid residues, respectively, the stability of the peptides in blood samples is a matter of concern. We reported previously that the stability of the immunoreactive BNP was affected by the material composing the blood collecting tubes [9,10]. We also suggested the participation of blood coagulation factors in the degradation of BNP because of its instability in blood stored in glass tubes.

Blood coagulation factor XII is activated when attached to negatively charged surfaces such as glass, kaolin, dextran sulfate, and sulfatide [11–14]. Once

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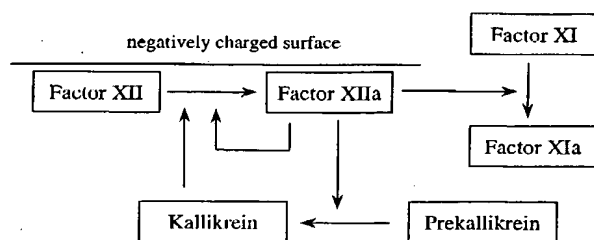


Fig. 1. Schematic models of activation of contact activation system.

factor XII is activated to factor XIIa, it converts plasma prekallikrein into kallikrein, which in turn activates factor XII to factor XIIa [15]. Factor XIIa also activates the zymogen of factor XIa [16]. This multi-enzyme system is called a 'contact activation system' (Fig. 1). Since the onset of cardiovascular disease involves the broad fields of coagulation, there may be a relationship between the formation and degradation of vasoreactive hormone and the activation of blood coagulation factors. As for ANP, some groups have reported results of *in vitro* and *in vivo* studies concerning its digestion [17–19]. However, there is little information about the protease which digests BNP to render it inactive.

In this study, we investigated the relationship between the degradation of BNP and the activation of blood coagulation factors, and found kallikrein to be the main factor in its degradation.

## 2. Materials and methods

### 2.1. Materials

Chemically-synthesized human ANP and human BNP were purchased from Peptide Institute (Minoh, Japan). Fluorescent kallikrein substrate, Val-Leu-Arg-AFC, and its end-product 7-amino-4-trifluoromethylcoumarin, were from Nova Biochem (Läufelfingen, Switzerland) and Sigma (St. Louis, MO), respectively. Acid-washed kaolin was purchased from Fisher Scientific (Fair Lawn, NJ). Blood collecting tubes made of siliconized glass or untreated glass, and polyethylene terephthalate (PET) were from Terumo (Tokyo, Japan). All other re-

agents were of the highest grade commercially available.

### 2.2. Blood samples

Venous blood samples from three healthy volunteers were collected into polypropylene tubes containing 1.5 g/l of EDTA-2Na. The blood was divided and plasma was prepared from half of the blood by centrifugation (2000×g, for 10 min). Human ANP and BNP were added (for a final concentration of ~200 ng/l) into whole blood and plasma, respectively, and then blood samples were pipetted into PET or glass tubes. The tubes were sealed and left standing at room temperature for 0, 2, 6, 24, and 72 h. After plasma separation from the whole blood samples, all specimens were stored at -80°C until measurement. The procedure was in accordance with the guidelines of the ethical committee of this institution.

### 2.3. Kaolin activation of plasma

Acid-washed kaolin (5 g/l plasma) was added to each plasma sample. After 1-h mixing of the plasma on a rotator at room temperature, kaolin was removed by centrifugation (2000×g, for 10 min).

### 2.4. Immunoassay

The ANP and BNP concentrations in each plasma sample were measured by sandwich radioimmunoassay using Shionoria ANP and BNP (Shionogi, Osaka, Japan), respectively. Each assay system consists of two different monoclonal antibodies which recognize the carboxyl terminal structure and the disulfide bond-mediated ring structure of ANP and BNP, respectively. A total of 100 µl of plasma was incubated with antibody-coated polystyrene beads and <sup>125</sup>I-labeled antibody. After washing of beads residual immunoreactivity was measured by a gamma-counter. Both assays required ~20 h to complete, and the measurable ranges were from 5 to 2000 ng/l for ANP and from 4 to 2000 ng/l for BNP.

### 2.5. Kallikrein activity

Kallikrein activity was measured by a fluorimetric

assay with Val-Leu-Arg-AFC as substrate [20–22]. Plasma (25  $\mu$ l) was diluted with 20 mmol/l Tris-HCl buffer, pH 8.0, containing 150 mmol/l of sodium chloride and 0.01% of bovine serum albumin to 400  $\mu$ l. The reaction was initiated with 100  $\mu$ l of 50  $\mu$ mol/l of AFC substrate. After incubation for 10 min at room temperature, the reaction was stopped by adding 25  $\mu$ l of 50% acetic acid. The generated fluorescence was measured with an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at 400-nm excitation and 505-nm emission wavelengths.

### 3. Results

Fig. 2 shows the stability of the immunoreactivity of chemically-synthesized natriuretic peptides added to the blood samples. No significant difference was observed in the stability of ANP among whole blood samples stored in the blood collecting tubes of different materials (Fig. 2a). ANP in plasma was more stable than in whole blood when it was stored in PET tubes (Fig. 3a). BNP lost its immunoreactivity faster than ANP both in whole blood and plasma samples when the samples were stored in the glass tubes, and the degradation of BNP in plasma was 2.2 times the rate observed in whole blood

(Figs. 2b and 3b). However, BNP was stabilized by storage of blood in the siliconized glass or PET tubes, and ~60% of the activity remained even after 72-h incubation of whole blood or plasma samples at room temperature. The stability of BNP in the whole blood and plasma were not different when the samples were stored in the PET or siliconized glass tubes.

To assess the activation of the blood coagulation factors, kallikrein activity in plasma was measured using fluorescent substrate. The enzyme activity in plasma stored in the glass tubes was increased although it was negligible in samples stored in the PET tubes (Fig. 4). Fig. 5 shows the stability of the immunoreactivity of BNP added to the kaolin-activated plasma or serum. The added BNP was decomposed completely in 6 h in kaolin-activated plasma, which was faster than the degradation rate of BNP added to serum samples.

### 4. Discussion

Our present data indicated that surface treatment of blood collecting tube delayed the degradation of BNP and that this treatment was less effective in stabilizing ANP (Figs. 2 and 3). These results suggest the difference of substrate specificity be-

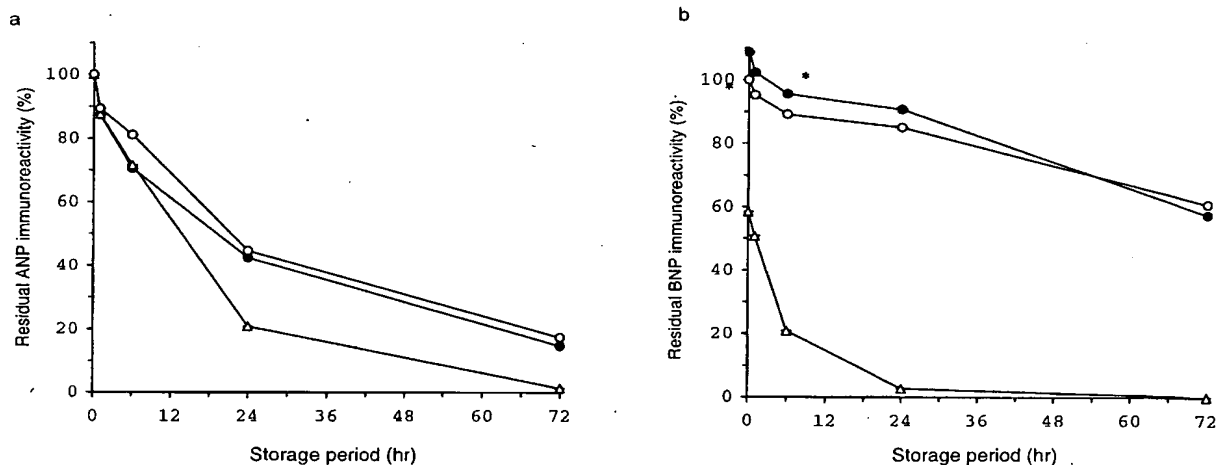


Fig. 2. Stability of the immunoreactivity of chemically synthesized (a) ANP and (b) BNP in whole blood samples. Peptide-spiked blood was stored in a glass tube  $\Delta$ , a siliconized glass tube  $\bullet$ , or a PET tube  $\circ$ . Data represent mean  $\pm$  S.D. ( $n=3$ ). \* $P<0.05$ .

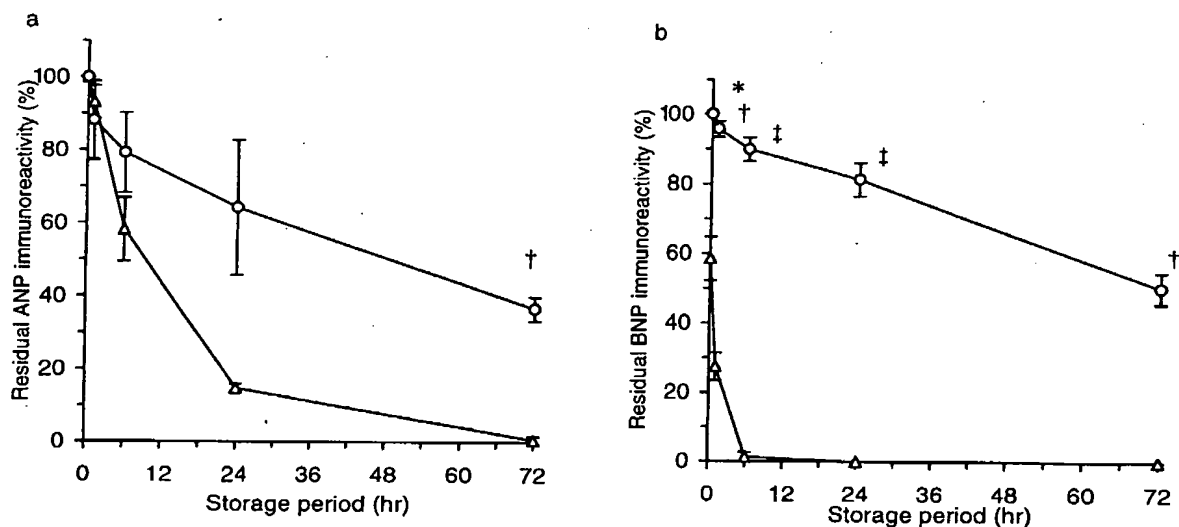


Fig. 3. Stability of the immunoreactivity of chemically synthesized (a) ANP and (b) BNP in plasma samples. Peptide-spiked plasma was stored in a glass tube  $\Delta$ , or a PET tube  $\circ$ . Data represent mean  $\pm$  S.D. ( $n=3$ ). \* $P<0.05$ , † $P<0.01$ , ‡ $P<0.001$  versus storage in the glass tubes, respectively.

tween ANP and BNP to proteases in blood samples. BNP lost its immunoreactivity in a shorter time in blood samples stored in glass tubes than in silicon-

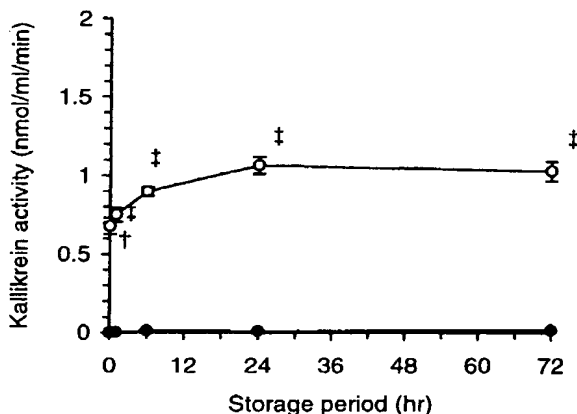


Fig. 4. Kallikrein activity in plasma stored in a glass tube  $\circ$ , or a PET tube  $\bullet$ . Data represent mean  $\pm$  S.D. ( $n=3$ ). † $P<0.01$ , ‡ $P<0.001$  versus storage in a PET tube, respectively.

ized glass or PET tubes. This implied that the protease (or proteases) activated by glass surface played an important role in digestion of BNP. Blood coagulation factors are candidates for agents that are activated by glass surfaces. In our study, kallikrein activity was increased in the plasma stored in the untreated glass tubes (Fig. 4). Also, the immunoreactivity of BNP was diminished rapidly when the peptide was added to kaolin-activated plasma or serum (Fig. 5a). The similarity of the structure between the fluorescent substrate of kallikrein used in this study (Val-Leu-Arg-AFC) and the carboxyl terminus of human BNP (Val-Leu-Arg-Arg-His-OH) strongly suggested the participation of kallikrein in the degradation of BNP in blood. Although kallikrein activity in plasma stored in the surface-treated tubes was negligible, ~40% of BNP immunoreactivity was lost after 72 h of incubation. Moreover, higher kallikrein activity was observed in serum than in kaolin-activated plasma, though immunoreactive BNP was more stable in serum than kaolin-activated plasma (Fig. 5b). Such discrepancy between the stability of BNP and kallikrein activity implies

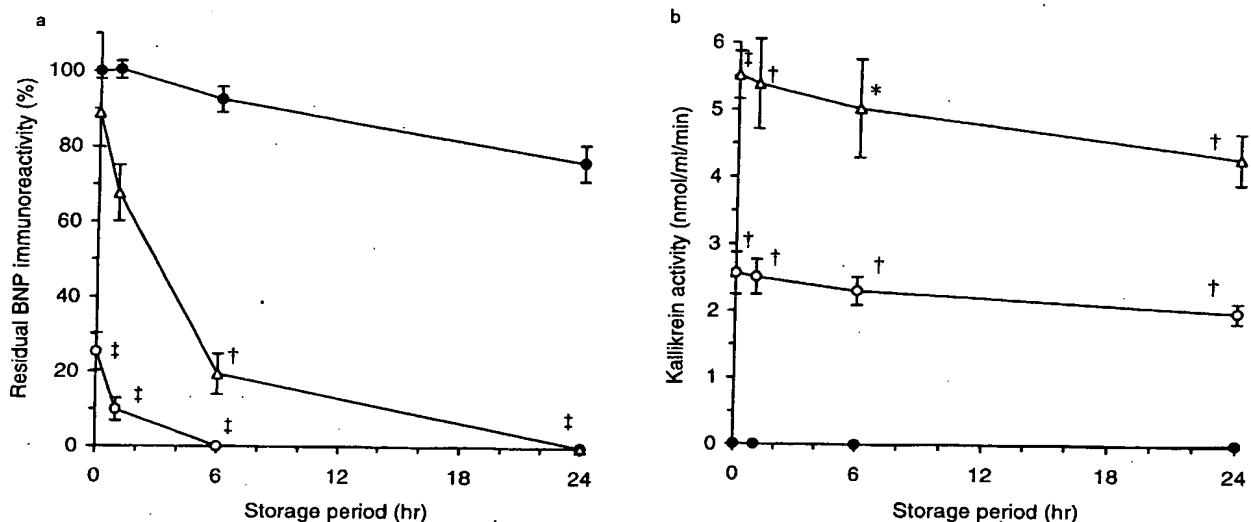


Fig. 5. (a) Stability of the immunoreactivity of chemically synthesized BNP in kaolin-activated plasma ○ and serum △. (b) Kallikrein activity in corresponding samples. Data represent mean  $\pm$  S.D. ( $n=3$ ). \* $P<0.05$ , † $P<0.01$ , ‡ $P<0.001$  versus non-treated plasma ●.

another protease might be involved in the degradation of the peptide. Some have suggested that neutral endopeptidase 24.11 (NEP), a membrane ectoenzyme widely distributed in the body and especially abundant in the kidney, lung, brain, and neutrophils [23–29], is involved in the degradation of ANP and BNP [17,23], however, further study is needed to determine the precise substrate specificity.

We previously reported that the stability of endogenous BNP was affected by the materials of blood collecting tubes [9]. In blood, BNP is present as proBNP (1–108), the mature form (BNP-32), and the N-terminal proBNP (BNP 1–76) [10]. Our BNP assay does not cross-react to the NT-proBNP form. Therefore, because the immunoreactivity of spiked BNP-32 was less stable than the endogenous BNP, these results indirectly suggest that pro-BNP is more stable in blood.

We suggest for the first time the relationship between BNP degradation and the blood coagulation factors. In patients with ischemic cardiovascular diseases such as unstable angina pectoris, intracoronary thrombus formation seems to be associated with activation of the contact activation system [30].

Further study is needed on the relationship between blood coagulation on intraluminal surface of blood vessel and degradation or metabolism of vasoreactive peptides in patients of cardiac diseases.

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# Erratum

## Erratum to "Degradation of human brain natriuretic peptide (BNP) by contact activation of blood coagulation system" [Clin. Chim. Acta 305 (2001) 181–186]<sup>☆</sup>

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The publisher regrets that in the above article, Fig. 2 was incorrect, and apologises for any confusion this may have caused. The correct figure is reproduced below.

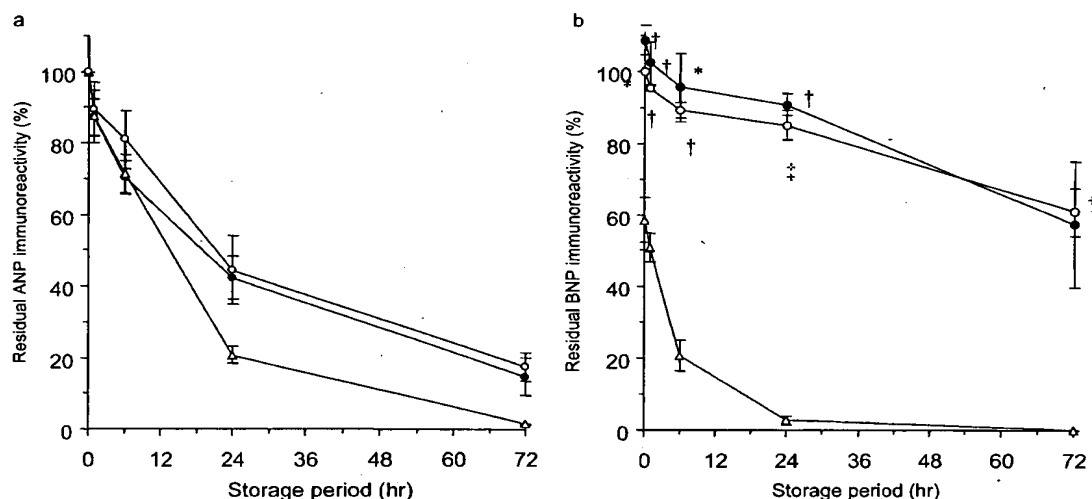


Fig. 2. Stability of the immunoreactivity of chemically synthesized (a) ANP and (b) BNP in whole blood samples. Peptide-spiked blood was stored in a glass tube  $\Delta$ , a siliconized glass tube  $\bullet$ , or a PET tube  $\circ$ . Data represent mean  $\pm$  S.D. ( $n = 3$ ). \*  $P < 0.05$ ; †  $P < 0.01$ ; ‡  $P < 0.001$  vs. storage in the glass tubes, respectively.

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Clinica Chimica Acta 285 (1999) 169–172



Short communication

## Stability of brain natriuretic peptide (BNP) in human blood samples

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### Abstract

Stability of immunoreactivity of human brain natriuretic peptide (BNP) in blood samples was investigated. After storage of the whole blood samples in the blood collecting tubes made of glass or polyethylene terephthalate (PET), residual immunoreactivity of BNP in the plasma was measured by sandwich radioimmunoassay for human BNP. BNP in the blood samples collected in the PET tubes were kept more stable than that in the glass tubes. The results suggested that commercially available PET tubes would enable more accurate BNP values and this would also help to simplify the sample preparation. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Brain natriuretic peptide (BNP); Immunoradiometric assay (IRMA); Heart failure

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### 1. Introduction

Brain natriuretic peptide (BNP) is a vasoreactive peptide hormone, which is synthesized and secreted mainly from the heart ventricles. The human plasma concentration of BNP is elevated with the decline of the function of the ventricle, making measurement of BNP concentration useful for the diagnosis of

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the patients with left ventricular dysfunction [1,2]. BNP is also a useful prognosis marker in cases of acute myocardial infarction or heart failure [3,4].

For accurate measurement of BNP concentration, suitable methods for sample preparation are indispensable. Although some groups have already reported on BNP stability in various blood samples, the results are still in dispute [5–8]. In our present study, we discovered that the stability of BNP immunoreactivity in the human blood samples was markedly affected by the material of the blood-collecting tubes themselves.

## **2. Materials and methods**

Blood-collecting tubes made of glass or polyethylene terephthalate (PET) were from Terumo (Tokyo, Japan). Both tubes are used in the clinical field and are commercially available (Venoject and Venoject II, respectively). Aprotinin from bovine lung was purchased from Sigma (St. Louis, MO, USA) and EDTA-2Na was from Dojin (Kumamoto, Japan).

Venous blood from four volunteers whose BNP values exceeded the cut-off value (18.4 ng/l) were collected and placed in the blood-collecting tubes made of glass or PET containing EDTA-2Na (1.5 g/l). Aprotinin (500 kKIU/l) was also added to some blood samples. The tubes were sealed and left standing for 0, 1, 6, 24, and 72 h at 4 or 25°C. After centrifugation (2000 g at 4°C), the plasma samples were stored at -80°C until BNP measurement. The BNP concentration in each plasma sample was measured by one-step sandwich radioimmunoassay using SHIONORIA BNP (Shionogi, Osaka, Japan) [9]. The assay system consists of two different monoclonal antibodies which recognize the carboxy terminal structure and the disulfide bond-mediated ring structure of BNP, respectively. The assay requires about 20 h to complete, and the measurable range of BNP is from 4 to 2000 ng/l. Only this assay system is sensitive enough to measure BNP concentration in clinical samples.

The procedure was in accord with the guidelines of the ethical committee of this institution.

## **3. Results**

Fig. 1 shows the stability of BNP immunoreactivity in the whole blood samples. The initial BNP value of each volunteer was 20.4, 33.5, 72.3, and 125 ng/l, respectively. When the whole blood samples were stored in the PET tubes with EDTA alone at 4°C for 72 h, 90.5% of the BNP immunoreactivity remained while 38.7% was lost on storage in the glass tubes even with aprotinin (Fig. 1(a)). As far as in the above conditions BNP stability was lower on storage

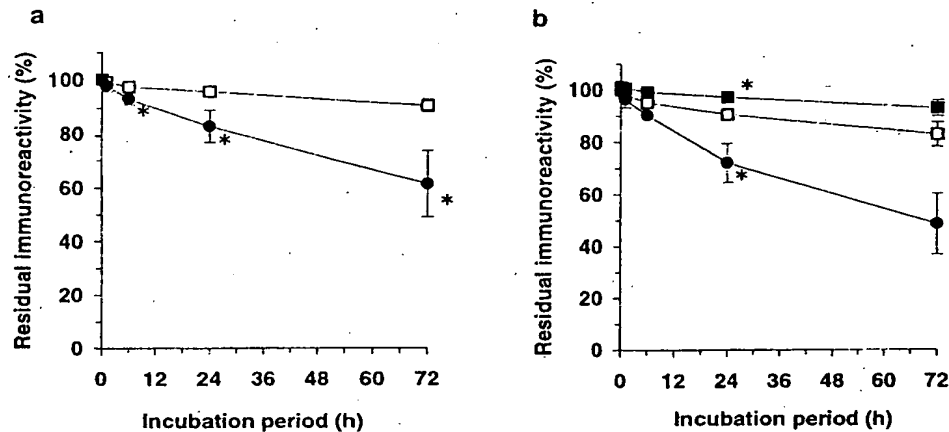


Fig. 1. Stability of BNP immunoreactivity in whole blood samples stored at 4°C (a) and 25°C (b). Whole blood samples containing EDTA-2Na (1.5 g/l blood) and aprotinin (500 kIU/l blood) were stored in ■ PET tubes or ● glass tubes. Whole blood containing EDTA alone was stored in □ PET tubes. The residual immunoreactivity was calculated by taking 100% as the initial BNP value of the sample collected into the PET tube containing EDTA. The data represent mean  $\pm$  SD (n=4). \*P<0.05 vs. EDTA alone in the PET tubes.

of these samples at room temperature (25°C) rather than at 4°C (Fig. 1(b)). Fig. 1(b) also shows that addition of aprotinin to the whole blood in the PET tubes effectively prevented BNP inactivation.

#### 4. Discussion

We discovered that the stability of BNP in the blood samples depended on the material of the tubes collecting human blood. We have previously reported that aprotinin, the serine protease inhibitor, is effective for stabilizing BNP in blood samples [8]. Fig. 1(a) shows, however, that BNP degradation could not be inhibited completely by the addition of aprotinin to the blood. On the other hand, the immunoreactivity of BNP in the whole blood samples was stable when the blood was stored in PET tubes. These results suggested the participation of activated protease, but further study is needed to elucidate the reason for this loss of stability.

Until now, not much attention has been directed to the materials of tubes collecting blood samples except for tests of coagulation factors. This finding better defines the collection and storage requirement for BNP measurement. Commercially available PET tubes would enable more accurate BNP values and

this would also help to simplify the sample preparation, both factors being essential to the clinical application of BNP measurement.

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